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(54) Title: PEPTIDES BINDING TO THE α2-MACROGLOBULIN RECEPTOR/LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN

(57) Abstract

The present invention relates to pharmaceutical compositions containing peptides capable of binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP) and a method for the prevention or treatment of indications involving interaction between the α2-MR/LRP and a lipoprotein or a lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase.

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PEPTIDES BINDING TO THE α2-MACROGLOBULIN RECEPTOR/LOW DENSITY LIPOPROTEIN RECEPTOR-RELATED PROTEIN

FIELD OF INVENTION

The present invention relates to pharmaceutical compositions containing peptides capable of binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein, as well as a method of using such peptides in therapy.

BACKGROUND OF THE INVENTION

Receptor-mediated uptake is an important step in lipoprotein metabolism and implicated in the pathogenesis of atherosclerosis. Lipoprotein lipase (LPL) can mediate cellular uptake of chylomicron remnants and very low density lipoprotein (VLDL) via proteoglycans and the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP). A model of LPL-mediated uptake of VLDL to α2-MR/LRP is shown in Fig. 1.

Lipoprotein lipase (LPL) is a non-covalent homodimer of 450 (bovine) or 448 (human) amino acid residues, the monomers each containing an N-terminal folding domain including the catalytic site, and a C-terminal folding domain (for a review, see 1). The α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP) consists of a membrane-spanning 85 kD β-chain and a 500 kD extracellular and ligand-binding α-chain (2,3). α2-MR/LRP binds and mediates the uptake of several unrelated ligands including α_2-macroglobulin/proteinase complexes through receptor-mediated endocytosis (3,4,5). Three established ligands are bound to other molecules at the cell surface before uptake via LRP: complex of plasminogen activator inhibitor-type 1 and urokinase-type plasminogen activator binds to the urokinase receptor (6), and LPL and apoE bind to proteoglycans (7,8,9).

The atherogenic (for a review, see 10) chylomicron remnants and VLDL are taken up through the α2-MR/LRP when associated with multiple LPL molecules (7,11) or when activated by apolipoprotein E (apoE) (12,13). Earlier studies have suggested LRP binding to the C-terminal domain of LPL (7). Recent results suggest that the LPL-mediated uptake through the α2-MR/LRP in cells of the arterial wall may be important for the pathogenesis of atherosclerosis. Firstly, LPL is expressed in subsets of smooth muscle cells and macrophages of human atherosclerotic lesions (14). Secondly, uptake of lipoprotein cholesteryl ester in arterial smooth muscle cells is markedly increased by the addition of LPL, but not apoE (15), and inbred mice exhibiting increased macrophage LPL secretion develop atherosclerosis (16). Finally, α_2-MR/LPL is expressed in the two cell types of the human arterial wall which may develop into foam cells; macrophages in atherosclerotic lesions express α2-MR/LRP and the scavenger receptor mediating uptake of oxidised LDL; smooth muscle cells in atherosclerotic lesions express α2-MR/LRP, but neither the scavenger receptor nor the LDL receptor.

SUMMARY OF THE INVENTION

In the course of research conducted to identify the α_2-MR/LRP binding site on LPL, it was found that a peptide which binds to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein is able to inhibit the uptake of VLDL in various types of cell, including fibroblasts from a patient with familial hypercholesterolemia and devoid of LDL receptors.

Accordingly, the present invention relates to a pharmaceutical composition comprising a peptide capable of binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP) such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in mammalian

cells, together with a pharmaceutically acceptable diluent or carrier.

In another aspect, the present invention relates to a method for the prevention or treatment of diseases or conditions involving interaction between the α2-MR/LRP and a lipoprotein or a lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in mammalian cells, the method comprising administering, to a patient in need thereof, an effective amount of a peptide capable of binding to the α2-MR/LRP so as to substantially inhibit said interaction between the α2-MR/LRP and the lipoprotein or the lipoprotein lipase or the lipoprotein/lipoprotein lipase complex.

In a further aspect, the present invention relates to the use of a peptide capable of binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP) such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, for the preparation of a medicament for the prevention or treatment of diseases or conditions involving interaction between the α2-MR/LRP lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in mammalian cells.

DETAILED DESCRIPTION OF THE INVENTION

In the pharmaceutical composition according to the invention, the peptide is preferably one which is capable of binding to α2-MR/LRP such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in cells expressing α2-MR/LRP, such as smooth muscle cells or macrophages.

According to the invention, the peptide is preferably a fragment of a lipoprotein lipase (LPL) or a functional homologue thereof.

The term "functional homologue" is intended to indicate a peptide derived from a homologous lipase, e.g. hepatic lipase, or a multiplicity of identical peptides coupled to a suitable carrier protein, or a peptide in which one or more amino acid residues have been substituted at one or more sites in the peptide sequence, or one or more amino acid residues have been deleted at one or more sites in the peptide sequence, or one or more amino acid residues have been inserted at one or more sites of the peptide sequence, provided that the peptide retains the ability to bind to the α2-MR/LRP and inhibit the uptake of lipoproteins into cells through this receptor. The peptide may also be a chemical derivative of an LPL fragment. An example of a suitable chemically derivatized peptide is hLPL 378-448 wherein SH groups (Cys^{418} and Cys^{438}) have been chemically blocked, e.g. by means of iodoacetamide. This derivative is active in terms of inhibiting LpL binding to α_2-MR/LRP.

A fragment of LPL capable of binding to the α2-MR/LRP according to the invention has been found in the C-terminal domain of LPL.

The parent LPL may suitably be derived from a variety of sources, such as from bovine, porcine, murine or human LPL. However, when the composition of the invention is intended for administration to human beings, the parent LPL is preferably of human origin. Examples of suitable peptides derived from human LPL are a peptide comprising amino acids 378-448, or amino acids 378-423 of human LPL.

The peptide included in the composition of the invention may for instance be prepared by peptide synthesis or by recombinant DNA techniques in a manner known per se.

According to one method, the peptide may be prepared by conventional methods of solution or solid phase peptide synthesis. For instance, solid phase synthesis may be carried out substantially as described by Stewart and Young, Solid Phase Peptide Synthesis, 2nd. Ed., Rockford, Illinois, USA, 1976. Solution peptide synthesis may for instance be carried out substantially as described by Bodansky et al., Peptide Synthesis, 2nd. Ed., New York, New York, USA, 1976. Peptide synthesis is particularly advantageous for the preparation of shorter peptides.

Alternatively, the peptide may be prepared by recombinant DNA techniques involving insertion of a DNA construct coding for the peptide into a suitable expression vector, transformation of a suitable host cell with the vector and cultivation of the transformed host cell under conditions permitting production of the peptide.

The DNA construct encoding the present peptide may be prepared synthetically by established standard methods, e.g. the phosphoamidite method described by S.L. Beaucage and M.H. Caruthers, Tetrahedron Letters 22, 1981, pp. 1859-1869, or the method described by Matthes et al., EMBO Journal 3, 1984, pp. 801-805. According to the phosphoamidite method, oligonucleotides are synthesized, e.g. in an automatic DNA synthesizer, purified, annealed, ligated and cloned in suitable vectors.

The DNA construct may also be of genomic or cDNA origin, for instance obtained by preparing a genomic or cDNA library and screening for DNA sequences coding for all or part of the LPL protein by hybridization using synthetic oligonucleotide probes in accordance with standard techniques (cf. Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor, 1989). In this case, a genomic or cDNA sequence encoding the entire LPL protein may be digested with one or more suitable restriction endonucleases, and a DNA fragment

encoding the desired peptide may be identified in a assay for binding to α2-MR/LRP as described in (7).

The DNA sequence encoding the peptide may be further modified at a site corresponding to the site(s) at which it is desired to introduce amino acid substitutions, e.g. by site-directed mutagenesis using synthetic oligonucleotides encoding the desired amino acid sequence for homologous recombination in accordance with well-known procedures. Examples of suitable modifications of the DNA sequence are nucleotide substitutions which do not give rise to another amino acid sequence of the present peptide, but which correspond to the codon usage of the host organism into which the DNA construct is introduced or nucleotide substitutions which do give rise to a different amino acid sequence and therefore, possibly, a different protein structure without, however, impairing the properties of the native variant. Other examples of possible modifications are insertion of one or more nucleotides into the sequence, or deletion of one or more nucleotides at either end or within the sequence.

The DNA construct encoding the present peptide may then be inserted into a suitable expression vector which may be any vector conveniently subjected to recombinant DNA procedures. The choice of vector will depend on the kind of host cell into which it is to be introduced. The vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

In the vector, the DNA sequence encoding the present peptide should be operably connected to a suitable promoter sequence (i.e. operably linked to the promoter sequence in the proper

reading frame). The promoter may be any DNA sequence which shows transcriptional activity in the host cell of choice and may be derived from genes encoding proteins either homologous or heterologous to the host cell.

Suitable promoters for use in prokaryotic host cells include, e.g. the promoter of the Bacillus stearothermophilus maltogenic amylase gene, Bacillus licheniformis α-amylase gene, Bacillus amyloliquefaciens BAN amylase gene, Bacillus subtilis alcaline protease gene, or Bacillus pumilus xylosidase gene, or by the phage Lambda P_R or P_L promoters, or the E. coli lac promoter, the ß-lactamase promoter (Villa-Kamaroff, et al., 1978, Proc. Natl. Acad. Sci. U.S.A. 75:3727-3731) and the tac promoter (DeBoer, et al., 1983, Proc. Natl. Acad. Sci. U.S.A. 80:21-25). Further references can also be found in "Useful proteins from recombinant bacteria" in Scientific American, 1980, 242:74-94.

Suitable promoters for use in yeast host cells include promoters from yeast glycolytic genes (Hitzeman et al., J. Biol. Chem. 255, 1980, pp. 12073-12080; Alber and Kawasaki, J. Mol. Appl. Gen. 1, 1982, pp. 419-434) or alcohol dehydrogenase genes (Young et al., in Genetic Engineering of Microorganisms for Chemicals (Hollaender et al, eds.), Plenum Press, New York, 1982), or the TPI1 (US 4, 599, 311) or ADH2-4c (Russell et al., Nature 304, 1983, pp. 652-654) promoters. Suitable promoters for use in filamentous fungus host cells are, for instance, the ADH3 promoter (McKnight et al., The EMBO J. 4, 1985, pp. 2093-2099) or the tpiA promoter. Examples of suitable promoters for directing the transcription of the DNA encoding the peptide of the invention in mammalian cells are the SV 40 promoter (Subramani et al., Mol. Cell Biol. 1, 1981, pp. 854-864), the MT-1 (metallothionein gene) promoter (Palmiter et al., Science 222, 1983, pp. 809-814) or the adenovirus 2 major late promoter.

The expression vector may also include other control sequences such as an operator, ribosome binding site, translation

initiation signal, and, optionally, a repressor gene or various activator genes. For instance, the DNA construct encoding the present peptide may be preceded by a ribosome binding site of of the Bacillus stearothermophilus maltogenic amylase gene, Bacillus licheniformis α-amylase gene, Bacillus amyloliquefaciens BAN amylase gene, Bacillus subtilis alcaline protease gene, Bacillus pumilus xylosidase gene, or E. coli lac gene.

The DNA sequence encoding the peptide of the invention may also be operably connected to a suitable terminator, such as (for mammalian cells) the human growth hormone terminator (Palmiter et al., op. cit.) or (for fungal hosts) the TPI1 (Alber and Kawasaki, op. cit.) or ADH3 (McKnight et al., op. cit.) promoters. The vector may further comprise elements such as polyadenylation signals (e.g. from SV 40 or the adenovirus 5 Elb region), transcriptional enhancer sequences (e.g. the SV 40 enhancer) and translational enhancer sequences (e.g. the ones encoding adenovirus VA RNAs).

To permit secretion of the expressed protein, a DNA sequence encoding a signal peptide may be inserted prior to the peptide-encoding sequence.

The recombinant expression vector may further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell expressing the SV40 T-antigen, e.g. COS-1 or COS-7 cells) is the SV 40 origin of replication or (when the host cell is a yeast cell) the yeast plasmid 2μ replication genes REP 1-3 and origin of replication. The vector may also comprise a selectable marker, e.g. a gene the product of which complements a defect in the host cell, e.g. the Schizosaccharomyces pombe TPI gene (described by P.R. Russell, Gene 40, 1985, pp. 125-130, or a gene which confers resistance to a drug, e.g. methotrexate, neomycin, hygromycin, ampicillin, kanamycin, chloramphenicol or tetracyclin.

The procedures used to ligate the DNA sequences coding for the present peptide, the promoter and the terminator, respectively, and to insert them into suitable vectors containing the information necessary for replication, are well known to persons skilled in the art (cf., for instance, Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, New York, 1989).

The host cell into which the expression vector of the invention is introduced may be any cell which is capable of producing the present peptide and is preferably a bacterial, yeast, fungal or mammalian cell.

The host cell used in the process of the invention may be any suitable bacterium which, on cultivation, produces large amounts of the desired peptide. Examples of suitable bacteria may be grampositive bacteria such as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus lentus*, *Bacillus brevis*, *Bacillus stearothermophilus*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus coagulans*, *Bacillus circulans*, *Bacillus lautus* or *Streptomyces lividans*, or gramnegative bacteria such as *Escherichia coli*. In *E. coli*, the peptide is typically produced in the form of inclusion bodies. The transformation of the bacteria may for instance be effected by protoplast transformation or by using competent cells in a manner known *per se* (cf. Sambrook et al., Molecular Cloning. A Laboratory Manual, 2nd Ed., Cold Spring Harbor, NY, 1989).

The yeast organism used as the host cell according to the invention may be any yeast organism which, on cultivation, produces large quantities of the present peptide. Examples of suitable yeast organisms are strains of the yeast species *Saccharomyces cerevisiae*, *Saccharomyces kluyveri*, *Schizosaccharomyces pombe* or *Saccharomyces uvarum*. The transformation of yest cells may for instance be effected by protoplast formation followed by transformation in a manner known *per se*.

Alternatively, fungal cells may be used as host cells of the invention. Examples of suitable fungal cells are cells of filamentous fungi, e.g. Aspergillus spp. or Neurospora spp., in particular strains of Aspergillus oryzae or Aspergillus niger. The use of Aspergillus oryzae for the expression of proteins is described in, e.g., EP 238 023.

Examples of suitable mammalian cell lines are the COS (ATCC CRL 1650), BHK (ATCC CRL 1632, ATCC CCL 10) or CHO (ATCC CCL 61) cell lines. Methods of transfecting mammalian cells and expressing DNA sequences introduced into the cells are described in e.g. Kaufman and Sharp, J. Mol. Biol. 159, 1982, pp. 601-621; Southern and Berg, J. Mol. Appl. Genet. 1, 1982, pp. 327-341; Loyter et al., Proc. Natl. Acad. Sci. USA 79, 1982, pp. 422-426; Wigler et al., Cell 14, 1978, p. 725; Corsaro and Pearson, Somatic Cell Genetics 7, 1981, p. 603, Graham and van der Eb, Virology 52, 1973, p. 456; and Neumann et al., EMBO J. 1, 1982, pp. 841-845.

The medium used to cultivate the cells may be any conventional medium suitable for growing bacterial, yeast, fungal or mammalian cells, depending on the choice of host cell. The peptide may be recovered from the culture medium by conventional procedures including separating the cells from the medium by centrifugation or filtration, if necessary after disrupting the cells to release an intracellular component (such as inclusion bodies), precipitating the proteinaceous components of the supernatant or filtrate by means of a salt, e.g. ammonium sulfate, purification by a variety of chromatographic procedures, e.g. ion exchange chromatography or affinity chromatography, or the like.

In the composition of the invention, the peptide may be formulated by any of the established methods of formulating pharmaceutical compositions, e.g. as described in Remington's Pharmaceutical Sciences, 1985. The composition may typically be

in a form suited for systemic injection or infusion and may, as such, be formulated with sterile water or an isotonic saline or glucose solution. The compositions may be sterilized by conventional sterilization techniques which are well known in the art. The resulting aqueous solutions may be packaged for use or filtered under aseptic conditions and lyophilized, the lyophilized preparation being combined with the sterile aqueous solution prior to administration. The composition may contain pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents and the like, for instance sodium acetate, sodium lactate, sodium chloride, potassium chloride, calcium chloride, etc. The concentration of the peptide in the composition may vary widely, i.e. from less than about 0.5%, such as from 1%, to as much as 15-20% by weight. A unit dosage of the composition may typically contain from about 0.1 to about 500 mg of the present peptide.

The present peptide is contemplated to be advantageous to use for therapeutic applications where inhibition of the interaction between LPL and α2-MR/LRP is desired. In particularly, the peptide is suggested for the prevention or treatment of atherosclerosis, in that the peptide has been found capable of inhibiting the uptake of lipoprotein into cells. The dosage of the peptide administered to a patient will vary with the type and severity of the condition to be treated, but is generally in the range of 1-100 mg/kg body weight.

The invention is further illustrated in the following examples which are not in any way intended to limit the scope of the invention as claimed.

DESCRIPTION OF THE DRAWINGS

The invention is further described with reference to the appended drawings, wherein

Fig. 1, top panel, is a proposed model for the interaction of LPL with the α2-MR/LRP α-chain, proteoglycans (pg) and triglyceride-rich lipoproteins (VLDL). The C-terminal domains of the LPL dimers are drawn in black. The clusters of complement- type repeats of the α2-MR/LRP α-chain are highlighted and the NPxY sequences of the β-chain, important for endocytosis via coated pits, are indicated. In the bottom panel, the LPL fragment (filled circle) competes with with LPL for binding to α2-MR/LRP (left, arrow) and for the heparan sulfate part of binding thought to occur via the C-terminal (middle, small arrow), whereas association of LPL with VLDL remains unperturbed (right).

Fig. 2 shows the binding of ^{125}I-α2-MR/LRP to fusion proteins containing the present peptides slot-blotted onto PVDF membranes, and lack of ^{125}I-VLDL binding to hLPL$^{378-448}$ containing fusion proteins.

Fig. 3 shows inhibition of LPL-mediated β-VLDL binding in CHO cells (top panel), inhibition of LPL-mediated β-VLDL uptake in Hep3b cells (middle panel) and FH fibroblasts in the presence of ^{14}C-oleate (bottom panel). Binding or uptake without additions is set at 100% (n=5). Error bars represent 1 S.D. (n=3) or range (broken line, n=2).

Example 1: **Production of recombinant fusion proteins containing C-terminal fragments of human lipoprotein lipase (hLPL)**

Plasmid construction. As template for producing nucleotide sequences encoding residues 378-423 and 378-448 of hLPL, a full length clone of hLpL (cloned into pUC18) was used. The cDNA fragments were amplified using the polymerase chain reaction (PCR) technique, as described by R.K. Saiki et al., Science 239, 1988, pp. 487-491. A BamHI restriction site and a sequence coding for the factor X substrate site was introduced at the 5'-end, and at the 3'-end a stop codon as well as a Hind III

restriction site was introduced. The reactions were carried out in 50 μl Taq polymerase buffer (Perkin Elmer) containing:

0.1 μg cDNA template

250 pmol N-terminal primer (5' -CACGGATCCATCGAGGGTAGGTTGAAGCTCAAATGGAAG- 3')

250 pmol C-terminal primer (5' -TTCAAGCTTAGCCTGACTTCTTATTCAG- 3' for the 378-423 fragment)

(5'-TTCAAGCTTACTCTCCTGCTTTTACTCT- 3' for the 378-448 fragment)

2.5 units Taq polymerase (Perkin Elmer)

250 nM of each four deoxynucleotides (dATP, dGTP, dTTP, dCTP)

The reaction mixture was overlaid with PCR oil and amplified on a Hybaid thermo cycler according to the following scheme:

Cycle 1-3:	Denaturation at 93°C for 1 minute
	Annealing at 50°C for 30 seconds
	Extension at 72°C for 30 seconds
Cycle 3-24:	Denaturation at 93°C for 1 minute
	Annealing at 60°C for 30 seconds
	Extension at 72°C for 30 seconds

The PCR products were extracted with phenol/chloroform, digested with 5 units BamHI (Amersham) and 5 units HindIII (Amersham) for one hour and purified on a 0.8% low melting agarose (Seaplaque GTG). The fragments were ligated (18) into the E. coli T7 expression vectors (19,20) pT7H6FX, pT7CIIH6FX and pT7CIIMLCH6FX. H6FX refers to the hexahistidine-Factor X substrate sequence MGSH6SIEGR. CII refers to the N-terminal 30 amino acids of the lambda CII phage protein, and MLC refers to the N-terminal 116 amino acids of chicken myosin light chain. The ligated products were transformed into XL1 Blue competent cells (Stratagene) and grown on an ampicillin (4μg/ml) agar plate (18). The plasmid constructs were amplified in 50 ml LB medium. An aliquot (1 ml) was stored at -80°C in 15% glycerol, and the rest was used for DNA purification using Quiagene's midi-prep kit.

Expression of recombinant peptides.

50 ml LB medium (supplemented with 4 μg ampicilin pr ml medium) was inoculated from the glycerol stock. After growing overnight at 37°C, 15 ml was added to 1 l of LB medium (supplemented with 4 μg ampicillin pr ml medium and 10 ml 1M $MgSO_4$) and further incubated at 37°C and 300 rpm until OD_{600}= 0.8. The expression in E. coli was then initiated with T7 lambda phage (21). After four hours of expression, the cells were harvested in a Sorvall GS-3 rotor after centrifugation for 10 min at 4000 rpm.

Purifiation of recombinant protein.

The cell pellet was resuspended in 30 ml 0.5 M NaCl, 50 mM TRIS-base and 10 mM EDTA, pH 8, and mixed with 50 ml of phenolsaturated with TRIS buffer. After strong sonication for 3 min, the phenol phase was separated by centrifugation in a Sorvall GS-3 rotor for 20 minutes at 8000 rpm. Protein was precipitated from the phenol phase by the addition of 2.5 volumes absolute ethanol and collected by centrifugation in a Sorvall GS-3 rotor for 10 minutes at 6000 rpm. The protein was resuspended in 25 ml 6 M guanidium hydrochloride, 50 mM TRIS-base, pH 8, and 0.1 M dithiotreitol. The buffer was changed to 8 M urea, 50 mM TRIS-base, pH 8, 0.5 M NaCl and 10 mM β-mecaptoethanol by use of a G25 gel filtration column (Pharmacia). The protein was loaded onto a Ni-NTA column (19,20) and washed with the above buffer until OD was constant. To allow disulfide reshuffling, 2 mM glutathion/0.2 mM oxidized glutathion was added, and the buffer was changed to 50 mM TRIS-base, pH 8, 0.5 M NaCl using a linear gradient. The recombinant protein was eluted with 0.5 M NaCl, 50 mM TRIS-base, pH 8, and 10 mM EDTA.

Example 2: **Assay for binding of LPL fragments to α_2MR/LRP.**

α_2MR/LRP is a two chain receptor with a 85 kDa membrane spanning β-chain and a 500 kDa non-covalently attached extracellular and ligand binding α-chain. Previous results have shown that purified ^{125}I-labeled α_2MR/LRP, either immobilized or in

solution, is capable of binding ligands with high affinity (7). This property provides the basis for procedures to measure binding of LPL fragments to the purified receptor. α_2MR/LRP was purified from human placenta and ^{125}I-labeled as described in detail (3,4,7).

Binding of ^{125}I-labeled purified α_2MR/LRP to immobilized fusion proteins containing fragments of human LPL. Slot blots of fragments (10 pmol), and of bovine LPL as a control (7), onto polyvinylidene difluoride (PVDF) membranes were performed using a Bio-RAD vacuum blotter. The membranes were blocked by incubation in buffer containing 5% bovine serum albumin, 150 mM NaCl, 2 mM $CaCl_2$, 50 mM Tris, pH 7.8, for 2 hours at 20°C, washed, and incubated with 50 pM ^{125}I-α_2MR/LRP for 16 hours at 4°C in 140 mM NaCl, 10 mM Hepes, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1% bovine serum albumin, pH 7.8 (buffer a). After washing, autoradiography (1-4 days) was performed using Hyperfilm (Amersham). The reaction is taken as a semiquantitative measure of binding activity of the immobilized fragment. ^{125}I-α_2MR/LRP bound to fusion proteins containing human $LpL^{378-448}$ and $LpL^{378-423}$, but not $LpL^{378-411}$ (cf. Fig. 2). Heparin (1 U/ml) abolished the binding. Since α_2MR/LRP does not bind heparin (3), this blocking is thought to be caused by heparin binding to the C-terminal fragment in agreement with previous results on chimers of lipoprotein lipase and hepatic lipase (22). In contrast to immobilized bovine LPL (7), none of the fusion proteins bound ^{125}I-labeled rabbit β-migrating very low density lipoproteins (β-VLDL) (cf. Fig. 2).

Inhibition of ^{125}I-α_2MR/LRP binding to immobilized bovine LPL. The apparent affinities of fusion proteins containing fragments of human LPL for binding to purified receptor were measured as the ability to inhibit binding of ^{125}I-α_2MR/LRP to immobilized bovine LPL. Microtiter wells (NUNC, Polysorp) were incubated for 2 hours at 20°C with bovine LPL in 50 mM $NaHCO_3$, pH 9.6, to provide about 500 fmol LPL per well. Following wash and blocking with 5% bovine serum albumin for 2 hours at 20°C,

incubations were performed in buffer a with 50 pM $^{125}I-\alpha_2MR/LRP$ and varying concentrations of fusion protein for 16 hours at 4°C. Following wash, the bound tracer was removed by the addition of 10% sodium dodecyl sulfate (SDS) and counted. About 25% of the added $^{125}I-\alpha_2MR/LRP$ was bound to the immobilized bovine LPL in the absence of inhibitor. The background achieved with high concentrations of LPL or fusion protein containing active LPL fragments corresponded to 0.5% of the added tracer and was not different from the blank value obtained in wells not coated with LPL. Half-maximal inhibition, taken as a measure of affinity, was about 200 nM for $C_{II}MLC-LpL^{378-448}$ and $C_{II}MLC-LpL^{378-423}$. This was not different from the apparent affinity of monomeric bovine LPL prepared by treatment with guanidinium hydrochloride (7).

Binding of LPL fragment to cellular α_2MR/LRP. Chemical crosslinking was used following previously published methods for demonstrating binding of bovine LPL (11). Confluent Hep3b cells in 6 cm Petri dishes were incubated with $^{125}I-C_{II}-LPL^{378-448}$ (190 nM, 1.2×10^6 cpm/ml) for 35 min at 4°C in the absence or presence of 1.3 μM bovine LPL. Following wash, the crosslinking reagent EDC-Sulfo NHS (Pierce) was added for 30 min at 22°C. The cells were centrifuged after washing steps, solubilized in 20 mM ethylmorpholin and 5% SDS followed by SDS-PAGE and autoradiography. $^{125}I-C_{II}-LPL^{378-448}$ bound to a protein corresponding to the location of α_2MR/LRP on the SDS gel, and the binding was abolished in the presence of excess fusion protein.

Example 3: **Inhibition of LpL-mediated binding and uptake of lipoproteins**

Rationale. LPL, concentrated on the cell surface via binding to heparan sulfate proteoglycans (1,7,9), can mediate cellular binding and uptake of triglyceride-rich lipoproteins since it is capable of binding to lipoprotein on one hand and to α_2MR/LRP on the other (7,11). Since the C-terminal LPL fragment bound to

purified and cellular receptor, but not to lipoprotein, it might function as an inhibitor of LPL-mediated lipoprotein uptake. Rabbit β-VLDL, a chylomicron remnant surrogate, was used as model lipoprotein (11-13).

Inhibition of LPL-mediated ^{125}I-β-VLDL binding. CHO cells were incubated as described previously for HepG2 cells (11) for 30 min at 4°C with ^{125}I-β-VLDL (1 μg protein/ml), 2 nM bovine LpL and with or without 1 μM C_{II}MLC-LPL$^{378-448}$. After washes, the cell surface bound ^{125}I-β-VLDL was released by heparin (770 U/ml) and counted. Parallel experiments in the absence of 2 nM bovine LPL was used to assess the minor ^{125}I-β-VLDL binding not mediated by LpL. The fusion protein was found to inhibit the LpL-mediated ^{125}I-β-VLDL binding by about 90% (Cf. Fig. 3, top panel).

Inhibition of LPL-mediated ^{125}I-β-VLDL uptake. Hep3b cells were were incubated for 90 min at 37°C with ^{125}I-β-VLDL (2 μg protein/ml), bovine LPL (2nM) and with or without 1 μM C_{II}-LPL$^{378-448}$ or C_{II}MLC-LPL$^{378-448}$. Parallel incubations were performed in the absence of 2 nM bovine LPL. The cells were washed, surface bound ligand was removed from the cells by heparin (770 U/ml), and the cells were lysed and counted for radioactivity. The fusion proteins containing LPL$^{378-448}$ were found to inhibit the LPL-mediated ^{125}I-β-VLDL uptake by 75-95% (Cf. Fig. 3, middle panel).

Inhibition of incorporation of ^{14}C-oleate induced by β-VLDL and LPL. Incorporation of labeled oleate into cholesterylesters induced by β-VLDL and LPL is an indirect measure of lipoprotein uptake by assessment of the acyl-CoA:cholesterol O-acyltransferase activity known to be induced by lipoprotein uptake and thereby cholesterol uptake (23). LDL receptor deficient fibroblasts from a patient with familial hypercholesterolemia (FH, Frensh-Canadian mutation) were used in these experiments and incubated as described (11). The FH fibroblasts were first incubated for 6 h at 37°C with 20 μg unlabeled β-VLDL protein/ml, 2 nM bovine LpL and with or

without 1 μM C_{II}-LPL$^{378-448}$ or C_{II}MLC-LPL$^{378-448}$. ^{14}C-oleate (0.4 μCi/ml, NEN) was then added for an additional 2 hour incubation at 37°C. Parallel incubations were performed in the absence of 2 nM bovine LPL. Following wash, the cellular lipids were extracted with heptane/isopropanol (3/2), applied to thin layer chromatography, and the cholesterolester spot was cut out and counted for radioactivity. The fusion proteins containing LPL$^{378-448}$ were found to inhibit the LPL induced incorporation of ^{14}C-oleate in the presence of β-VLDL by 60-80% (cf. Fig. 3, bottom panel).

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CLAIMS

1. A pharmaceutical composition comprising a peptide capable of binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP) such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in mammalian cells, together with a pharmaceutically acceptable diluent or carrier.

2. A pharmaceutical composition according to claim 1, wherein the peptide is a fragment of a lipoprotein lipase (LPL) or a functional homologue thereof.

3. A pharmaceutical composition according to claim 2, wherein the peptide is part of the C-terminal domain of LPL.

4. A pharmaceutical composition according to claim 1, wherein the peptide is one which is capable of binding to α2-MR/LRP such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in cells expressing α2-MR/LRP, such as smooth muscle cells or macrophages.

5. A pharmaceutical composition according to claim 3, wherein the LPL is human LPL.

6. A pharmaceutical composition according to claim 5, wherein the peptide comprises amino acids 378-448 of human LPL.

7. A pharmaceutical composition according to claim 5, wherein the peptide comprises amino acids 378-423 of human LPL.

8. A pharmaceutical composition according to any of claims 1-7 for the prevention or treatment of atherosclerosis.

9. A pharmaceutical composition according to any of claims 1-8, which comprises 0.1-500 mg of the peptide.

10. A method for the prevention or treatment of diseases or conditions involving interaction between the α2-MR/LRP and a lipoprotein or a lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in mammalian cells, the method comprising administering, to a patient in need thereof, an effective amount of a peptide capable of binding to the α2-MR/LRP so as to substantially inhibit said interaction between the α2-MR/LRP and the lipoprotein or the lipoprotein lipase or the lipoprotein/lipoprotein lipase complex.

11. A method according to claim 10, wherein the peptide is a fragment of a lipoprotein lipase (LPL) or a functional homologue thereof.

12. A method according to claim 11, wherein the peptide is part of the C-terminal domain of LPL.

13. A method according to claim 10, wherein the peptide is one which is capable of binding to α2-MR/LRP such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in cells expressing α2-MR/LRP, such as smooth muscle cells or macrophages.

14. A method according to claim 11, wherein the LPL is human LPL.

15. A method according to claim 14, wherein the peptide comprises amino acids 378-448 of human LPL.

16. A method according to claim 14, wherein the peptide comprises amino acids 378-423 of human LPL.

17. A method according to any of claims 10-16 for the prevention or treatment of atherosclerosis.

18. A method according to any of claims 10-17, wherein the effective amount of the peptide is in the range of 0.1-100 mg/kg body weight.

19. Use of a peptide capable of binding to the α2-macroglobulin receptor/low density lipoprotein receptor-related protein (α2-MR/LRP) such as to inhibit any interaction between the α2-MR/LRP and a lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, for the preparation of a medicament for the prevention or treatment of diseases or conditions involving interaction between the α2-MR/LRP lipoprotein or lipoprotein lipase or a complex of a lipoprotein and a lipoprotein lipase, said interaction resulting in the uptake of lipoprotein in mammalian cells.

20. Use according to claim 19, wherein the peptide is a fragment of a lipoprotein lipase (LPL) or a functional homologue thereof.

21. Use according to claim 20, wherein the peptide is part of the C-terminal domain of LPL.

22. Use according to claim 21, wherein the LPL is human LPL.

23. Use according to claim 22, wherein the peptide comprises amino acids 378-448 of human LPL.

24. Use according to claim 22, wherein the peptide comprises amino acids 378-423 of human LPL.

25. Use according to any of claims 19-24 for the prevention or treatment of atherosclerosis.

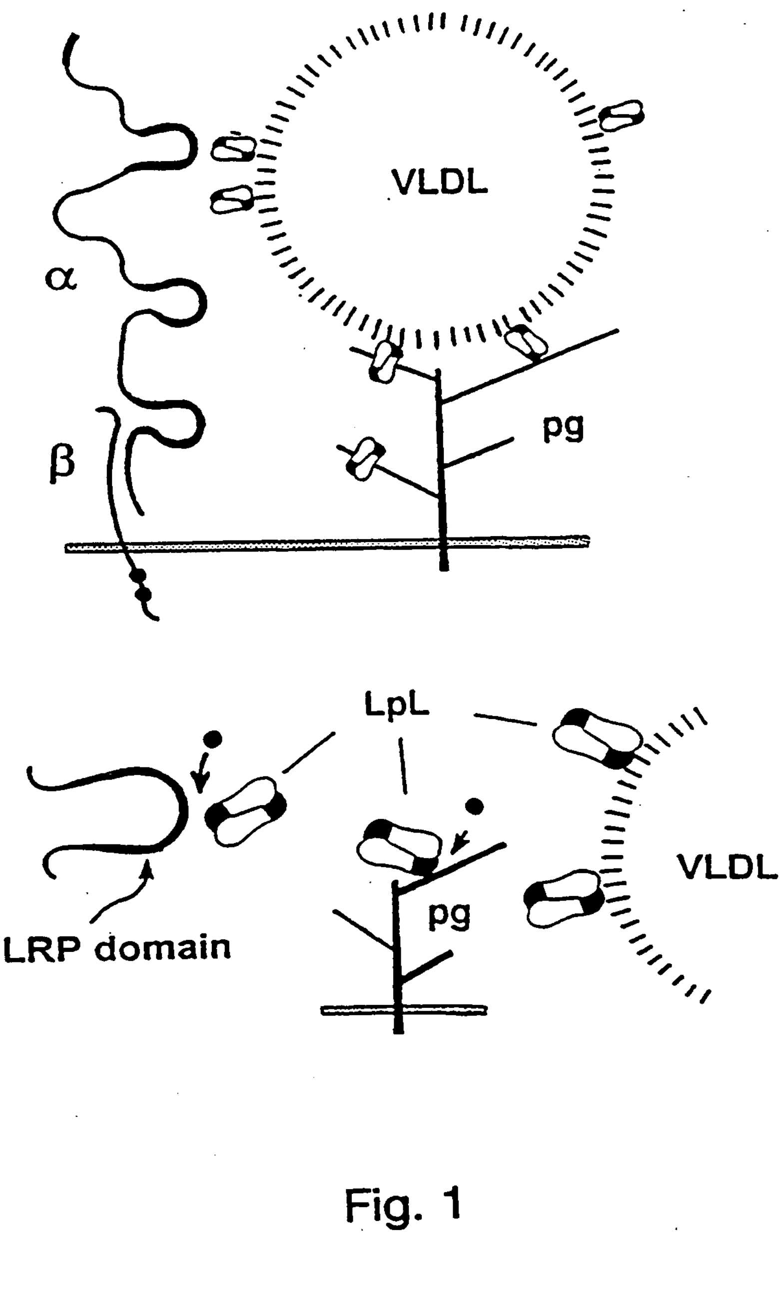

Fig. 1

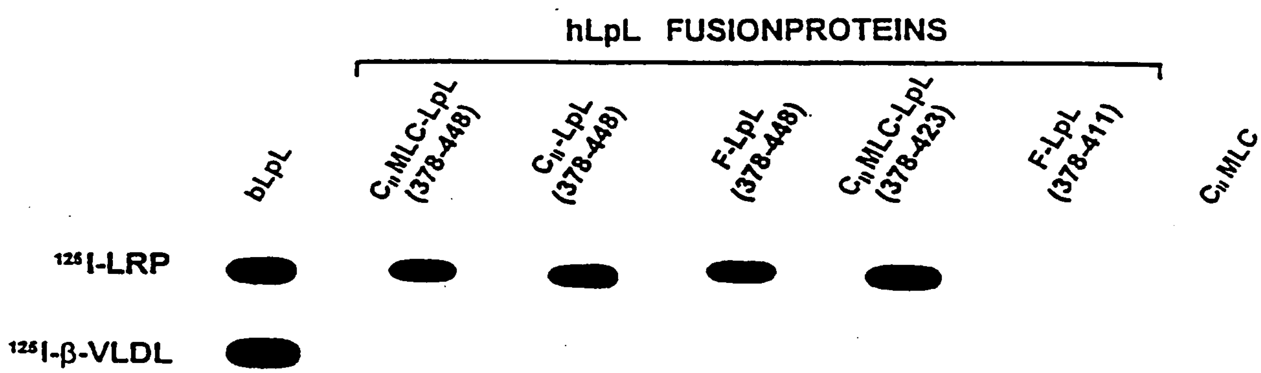
hLpL FUSIONPROTEINS
bLpL
CII MLC-LpL (378-448)
CII-LpL (378-448)
F-LpL (378-448)
CII MLC-LpL (378-423)
F-LpL (378-411)
CII MLC
125I-LRP
125I-β-VLDL

Fig. 2

3/3

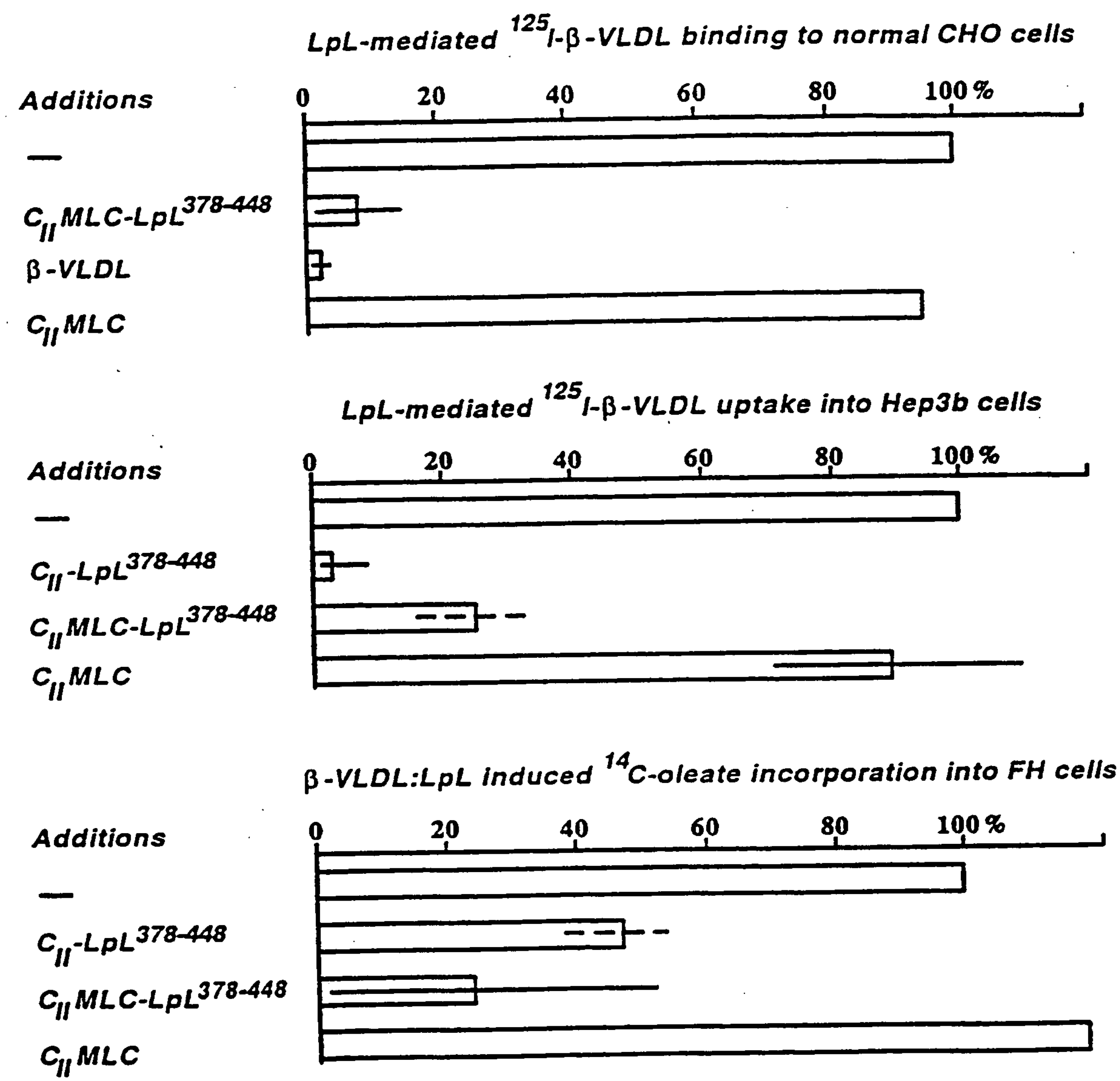
LpL-mediated 125I-β-VLDL binding to normal CHO cells
Additions
0
20
40
60
80
100 %
—
CIIMLC-LpL378-448
β-VLDL
CIIMLC
LpL-mediated 125I-β-VLDL uptake into Hep3b cells
Additions
0
20
40
60
80
100 %
—
CII-LpL378-448
CIIMLC-LpL378-448
CIIMLC
β-VLDL:LpL induced 14C-oleate incorporation into FH cells
Additions
0
20
40
60
80
100 %
—
CII-LpL378-448
CIIMLC-LpL378-448
CIIMLC

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00161

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: A61K 38/46

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, EMBASE, WPI, WPIL, US PATENTS FULLTEXT DATABASES, SCISEARC
H

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	Dialog Information Service, file 155, Medline, Dialog accession No. 09151126, Medline accession No. 95081126, Nykjaer A. et al: "A carboxyl-terminal fragment of lipoprotein lipase binds to the low density lipoprotein receptor-related protein and inhibits lipase-mediated uptake of lipoprotein in cells", J Biol Chem (UNITED STATES) Dec 16 1994, 269 (50) p 31747-55 --	1-9,19-25

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" erlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 July 1995	25 -07- 1995
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Carolina Palmcrantz Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00161

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Dialog Informatiion Service, file 155, Medline, Dialog accession No. 08605483, Medline accession No. 93315483, Nykjaer A. et al: "The alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein binds lipoprotein lipase and beta-migrating very low density lipoprotein associated with the lipase", J Biol Chem (UNITED STATES) Jul 15 1993, 268 (20) p 15048-55 --	1-9,19-25
X	Dialog Information Service, file 155, Medline, Dialog accession No. 07904002, Medline accession No. 92042002, Herz J. et al: "39-kDa protein modulates binding of ligands to low density lipoprotein receptor-related protein/alpha 2-macroglobulin receptor", J Biol Chem (UNITED STATES) Nov 5 1991, 266 (31) p 21232-8 --	1,4,8-9,19, 25
A	Dialog Information Service, file 155, Medline, Dialog accession No. 07882849, Medline accession No. 92020849, Beisiegel U. et al: "Lipoprotein lipase enhances the binding of chylomicrons to low density lipoprotein receptor-related protein", Proc Natl Acad Sci USA (UNITED STATES) Oct 1 1991, 88 (19) p 8342-6 --	1-9,19-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 95/00161

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 10-18
because they relate to subject matter not required to be searched by this Authority, namely:
See PCT Rule 39.1 (iv): Methods for treatment of the human or animal body by therapy.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.